STUDIES ON THE ISOLATED ISLET TISSUE OF FISH. IV. IN VITRO INCORPORATION OF C^{14}- AND H^3-LABELED AMINO ACIDS INTO GOOSEFISH ISLET TISSUE PROTEINS [1, 2]

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Elucidation of the factors involved in the biosynthesis of insulin is essential for a better understanding of the etiology and development of diabetes mellitus. To this end, *in vitro* studies of the incorporation of labeled amino acids into islet cell proteins have been undertaken. In mammalian species the islet tissue is dispersed into a million or more individual islets of Langerhans, whose total mass approximates only 1% of the pancreatic mass. This makes it very difficult to separate the islet from the acinar tissue. In teleost fish, however, the islet tissue is concentrated into one or more discrete bodies called the principal islets (Diamare, 1905; Rennie, 1905); the acinar tissue, in contrast to that of mammalian species, is dispersed throughout the mesentery and located along the bile duct and within the liver. The goosefish, *Lophius piscatorius,* a marine teleost of wide distribution, was chosen because of its large and discrete aggregations of islet tissue, which are rich in extractable insulin and relatively free of exocrine tissue (Macleod, 1922). The *in vitro* study of insulin biosynthesis, using isolated islet tissue and radioactive amino acids, is based on the assumption that this process is closely analogous to the natural biosynthetic pathway. Net synthesis of specific proteins from labeled amino acids, *in vitro,* has been established for serum albumin (Peters and Anfinsen, 1950) and cytochrome *c* (Kalf *et al.,* 1959).

Previous *in vitro* studies of insulin biosynthesis using labeled amino acids and mammalian pancreas have been carried out by Pettinga and Rice (1952), Vaughan and Anfinsen (1954), and Light and Simpson (1956). Since the ratio of exocrine to islet tissue is large in mammalian pancreas, it was necessary to use large samples of tissue (weighing 10 to 100 gm.). In addition, the presence of large amounts of non-insulin proteins required the use of exacting purification procedures.

The present report deals with our studies on the *in vitro* incorporation of C^{14}-labeled amino acids into proteins using the isolated islet tissue of the goosefish. A preliminary report of these studies has been published (Bauer and Lazarow, 1961).

[1] These investigations were initiated in the summer of 1959 by the late Austin Lloyd Yates, a brilliant young graduate student at the University of Minnesota. Mr. Yates had successfully demonstrated that C^{14}-labeled amino acids, added to toadfish islet tissue *in vitro,* were incorporated into the alcohol-soluble fraction. Because of his tragic accidental death in the summer of 1959, and because all of the original protocols were lost, these studies were interrupted; they were resumed in June, 1960.

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Methods

Incubation procedure. Goosefish were obtained periodically during the summer months in the vicinity of Woods Hole, Massachusetts, and maintained in refrigerated sea water tanks. The large principal islet, located in the mesentery adjacent to the cystic duct, was removed, and the connective tissue capsule dissected away. Occasionally, the smaller secondary islet, located near the pyloric stomach, was also used. One to three pieces of islet tissue, weighing between 2 and 6 mg., were incubated at 25° C. in a micro-homogenizer tube (Fig. 1). The incubation medium

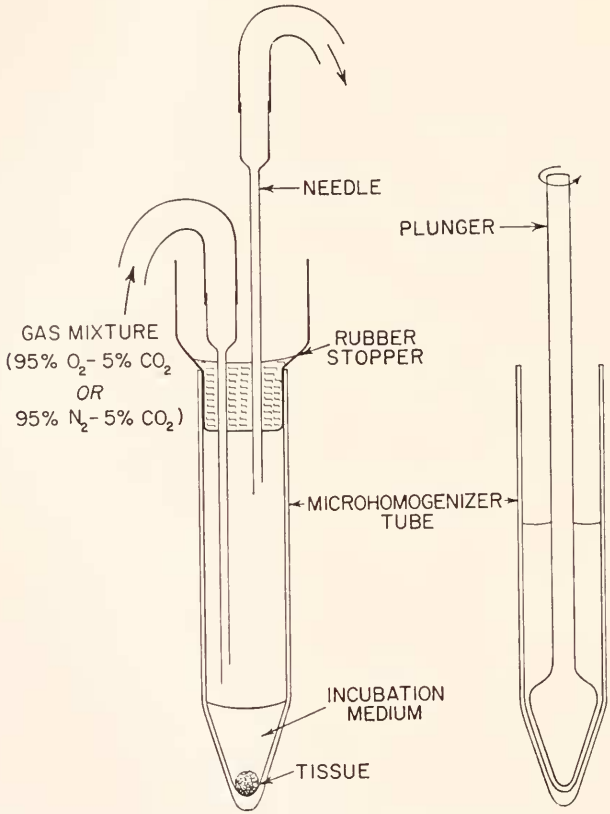

Figure 1. The incubation and extraction vessel.

consisted of 50 μl. of Krebs' Ringer bicarbonate buffer (Krebs and Eggleston, 1940), containing leucine-C^{14} + valine-C^{14}, or leucine-H^3 (initial pH 7.4). In experiments using C^{14}-labeled amino acids, the amino acids were dissolved in water at 11 times the desired final concentration. One part of this mixture was added to 10 parts of mammalian Krebs' Ringer bicarbonate buffer to give a final electrolyte concentration of 0.14 *M*; this is isotonic to fish blood. (Since there are 6 leucine and 5 valine residues per mole of insulin, a mixture of leucine-C^{14} and valine-C^{14} was

used in order to obtain high specific activities of the proteins synthesized.) When leucine-H^3 was used, the desired amount of amino acid was dissolved in the appropriate volume of 0.14 *M* Krebs' Ringer bicarbonate buffer.

The concentrations of amino acids used in these studies were the following: DL-leucine-1-C^{14}, 2.1 m*M*/l. (specific activity = 8.75 mC./m*M*), or 2.2 m*M*/l. (S. A. = 7.80 mC./m*M*); DL-valine-1-C^{14}, 1.6 m*M*/l. (S. A. = 10.5 mC./m*M*), or 3.0 m*M*/l. (S. A. = 6.05 mC./m*M*); DL-leucine-4,5-H^3, 0.43 m*M*/l. (S. A. = 3,570 mC./m*M*).

The micro-homogenizer tubes, containing the incubation medium, were equilibrated with the appropriate gas mixture and stoppered. They were re-equilibrated after introduction of the tissue and intermittently during the incubation period. Mixtures of 95% O_2-5%CO_2 were used in aerobic experiments, or 95% N_2-5%CO_2 in anaerobic experiments. Before introduction into the vessels, the gas mixtures were saturated with water vapor by bubbling them through a series of flasks containing water, thus minimizing evaporation of medium from the incubation tubes. The micro-homogenizer tubes were shaken periodically throughout the incubation period.

Extraction procedure. The incubation period was terminated by adding an equal volume (50 μl.) of 10% trichloroacetic acid (TCA). An additional 100 μl. of 5% TCA were added. The plunger of each micro-homogenizer was inserted and the tissue homogenized. The plunger was removed and the tubes centrifuged for 4 minutes at 3000 r.p.m. in a clinical centrifuge, and the supernatant decanted (by capillary pipette). Subsequent washings and extractions were carried out in the original micro-homogenizer tube, the plunger being used to re-suspend precipitates. The tubes were cooled in an ice bath during the extraction and purification procedures.

The precipitate was washed with 200 μl. of 5% TCA (5 times) in order to remove free amino acids. The washed residue was extracted with 200 μl. of 95% ethanol; this was repeated once, and followed by a 95% ethanol-ether (1 : 1), and an ether wash. The residual protein, designated as the trichlor-precipitable protein residue (TPR), was suspended in 500 μl. of acetone, and aliquots of this were immediately transferred to planchets for counting. The term "protein" includes nucleoprotein precipitated by cold TCA.

Purification procedure. The supernatant of the first alcohol extraction, designated as the first alcohol-soluble fraction (ASF), was transferred to a new micro-homogenizer, and the alcohol removed by evaporation under a stream of nitrogen. The residue was washed twice with acetone (100 μl.), twice with ether (200 μl.), and then dissolved in 100 μl. of acid alcohol (75% ethanol acidified with HCl; pH < 1.0). After centrifugation, the supernatant was removed and the residue re-extracted with 100 μl. of acid alcohol. The acid alcohol supernatants were combined and transferred to a new micro-homogenizer. Four volumes of acetone were added; following centrifugation and decantation, the precipitate was redissolved in acid alcohol and aliquots transferred to planchets for counting.

The acetone and ether supernatants obtained were pooled and plated *in toto;* this fraction is designated as the lipid solvant fraction (LSF). The acetone supernatant was also plated *in toto*. The radioactivity in this fraction represents unprecipitated proteins and soluble peptides.

Insulin-I^{131}. The I^{131}-labeled insulin used in the recovery experiments was obtained from Abbott Laboratories; it had a specific activity of 2 to 5 mC./mg.

Radioisotope counting. In the experiments using C^{14}-labeled amino acids, triplicate 25-μl. aliquots were transferred to planchets and air-dried. The samples were counted to an accuracy of 5%, using a gas flow counter (Nuclear Chicago) under conditions in which the counting efficiency was 31%. In the studies using tritium, triplicate 25-μl. aliquots were pipetted into the counting vessels; 1 ml. of hyamine and 10 ml. of toluene containing 0.4% PPO (2,5-diphenyloxazole) were added. The samples were counted to an accuracy of 5% in a Packard Tri-Carb liquid scintillation counter at an efficiency of about 4%.

Results

The distribution of radioactivity recovered in the various fractions during the washing and extraction procedures is illustrated in Table I. The counts re-

Table I

Distribution of radioactivity in various fractions isolated from goosefish islet tissue incubated 2 hours in C^{14}-labeled leucine and valine

Step	Fraction	cpm†	%*	%**
A	TCA supernatant	1,460,000	96.7	
B	First TCA wash	41,500	2.8	
C	Second TCA wash	2,290	.15	
D	Third TCA wash	192	.01	
E	Fourth TCA wash	83	<.01	
F	Fifth TCA wash	54	<.01	
G	First alcohol-soluble fraction (ASF)	1,860	.12	31.4
H	Second alcohol-soluble fraction (ASF-2)	232	.02	3.9
I	Ethanol-ether fraction	25	<.01	.42
J	Ether fraction	14	<.01	.21
K	Trichlor-precipitable protein residue (TPR)	3,790	.25	64.0

† Expressed as cpm/200 μl. (total supernatant).
* % of total counts recovered.
** % of residual counts remaining after last TCA wash.

maining in the fifth TCA wash, presumably due to the presence of free C^{14}-amino acids, were 50 cpm/200 μl. in this experiment. This is only 3% of the counts recovered in the ASF. The counts in the ethanol-ether and ether extractions were 25 and 14 cpm/200 μl., respectively; these are less than 2% of the counts recovered in the TPR.

Recovery experiments were carried out in which insulin labeled with I^{131} was added to the incubation medium, and the extraction procedure followed. The insulin-I^{131} was added to the incubation medium along with islet tissue; the C^{14}-labeled amino acids were omitted. The results, summarized in Table II, indicate that 79 to 89% of the added radioactivity was recovered in the ASF at 0 hours of incubation (*i.e.*, when TCA was added immediately after the addition of insulin-I^{131} to the incubation medium). When insulin-I^{131} was incubated with islet tissue

TABLE II

Recovery of I^{131}-labeled insulin added to islet tissue

Experiment	Incubation time (hours)	Alcohol-soluble fraction (ASF)		TCA supernatant		Trichlor-precipitable protein residue (TPR)	
		cpm	% of total	cpm	% of total	cpm	% of total
1	0	125,000	89	8,980	6.4	8,100	5.8
2a (Av. of 2 samples)	0	105,000	79	13,500	10	14,500	11
2b	1	79,400	62	26,500	21	19,600	15
2c	2	64,300	49	49,000	37	17,600	13

Each vessel contained approximately .289 μC. of I^{131}-labeled insulin and 1.44 to 1.91 mg. of goosefish islet tissue.

for one and two hours, the counts recovered in the ASF decreased to 62 and 49%, respectively. This was associated with a corresponding increase in counts recovered in the TCA-soluble radioactivity. These data suggest that the added bovine insulin is partially degraded in the presence of islet tissue.

In a similar experiment, not illustrated, the counts in both the first alcohol-soluble (ASF) and second alcohol-soluble (ASF-2) fractions were determined; 80% of the added counts were recovered in the first, and about 9% in the second.

TABLE III

Incorporation of leucine-C^{14} + valine-C^{14} into goosefish islet and liver fractions

Tissue		0-hour control				2-hour incubation				Ratio $\frac{\text{ASF}}{\text{TPR}}$
		Alcohol-soluble fraction (ASF)		Trichlor-precipitable protein residue (TPR)		Alcohol-soluble fraction (ASF)		Trichlor-precipitable protein residue (TPR)		
		No. of Samples	cpm†	No. of Samples	cpm†	No. of Samples	cpm†	No. of Samples	cpm†	
Islet	A*	10	326	10	150	22	5,900	22	7,440	0.79
	B**	2	96	2	131	3	3,860	3	6,480	0.61
Liver	A*	2	0	2	248	3	1,930	3	8,800	0.22
	B**	2	42	2	246	3	1,220	3	6,090	0.20

† The values given represent the average counts per minute/10 mg. of tissue.
* The concentration of leucine-C^{14} was 2.1 mM/l., S. A. = 8.75 mC./mM.
The concentration of valine-C^{14} was 1.6 mM/l., S. A. = 10.5 mC./mM.
** The concentration of leucine-C^{14} was 2.2 mM/l., S. A. = 7.80 mC./mM.
The concentration of valine-C^{14} was 3.0 mM/l., S. A. = 6.05 mC./mM.

TABLE IV

Effect of incubation time on leucine-C^{14} + valine-C^{14} incorporation into protein fractions of goosefish islet tissue

Time of incubation (hours	cpm/10 mg. of tissue: Alcohol-soluble fraction (ASF)	cpm/10 mg. of tissue: Trichlor-precipitable protein residue (TPR)
0	390	210
½	2,740	3,030
1	3,870	6,650
2	10,400	9,450
4	15,200	25,400
6	25,400	35,800
9	23,500	50,500
12	25,300	46,000

The concentration of leucine-C^{13} was 2.2 m*M*/l., S. A. = 7.80 mC./m*M*.
The concentration of valine-C^{14} was 3.0 m*M*/l., S. A. = 6.05 mC./m*M*.

On the basis of these recovery experiments, it was presumed that insulin present in islet tissue would likewise appear in the ASF. This assumption is consistent with the known solubility of insulin in acid alcohol (the pH of the ASF was approximately 2; this acidity is due to the presence of TCA extracted from the precipitate by alcohol). In similar experiments, where C^{14}-labeled amino acids were added to the incubation medium, the majority of the counts extracted by alcohol were likewise found in the first ASF.

A comparison of C^{14}-amino acid radioactivity incorporated into the various fractions of islet and liver is shown in Table III. The total number of counts incorporated into the ASF of islet tissue was approximately equal to that found in the TPR; the ratios of counts in the ASF/TPR were 0.79 and 0.61. By

TABLE V

Effect of aerobic and anerobic conditions on the incorporation of leucine-C^{14} + valine-C^{14} into goosefish islet tissue protein fractions

Experiment	Time of incubation (hours)	Alcohol-soluble fraction (ASF): No. of samples	cpm/10 mg. tissue: Aerobic	cpm/10 mg. tissue: Anaerobic	% inhibition	Trichlor-precipitable protein residue (TPR): No. of samples	cpm/10 mg. tissue: Aerobic	cpm/10 mg. tissue: Anaerobic	% inhibition
A*	0	2	113	113		2	383	383	
	2	3	2,900	239	91.8	2–3	4,500	1,450	67.8
B**	0	1	124	124		1	44.5	44.5	
	2	3	3,820	814	78.7	2–3	7,130	2,260	68.2

* The concentration of leucine-C^{14} was 2.1 m*M*./l., S. A. = 8.75 mC./m*M*. The concentration of valine-C^{14} was 1.6 m*M*./l., S. A. = 10.5 mC./m*M*.

** The concentration of leucine-C^{14} was 2.2 m*M*./l., S. A. = 7.80 mC./m*M*. The concentration of valine-C^{14} was 3.0 m*M*./l., S. A. = 6.05 mC./m*M*.

contrast, the total number of counts incorporated into the ASF of liver was much smaller than that in the TPR (ASF/TPR ratios of 0.20 and 0.22). Thus, islet tissue appears to incorporate a much greater fraction of the added counts into the protein component which is soluble in alcohol.

The effect of incubation time on the incorporation of amino acid radioactivity into the ASF and the TPR fractions is shown in Table IV. The counts incor-

TABLE VI

Effect of varying C^{14}-amino acid concentration on incorporation into goosefish islet fractions

Experiment	Sample	C^{14}-amino acid concentration mM/l.†	mg. of tissue	Alcohol-soluble fraction (ASF)		Trichlor-precipitable protein residue (TPR)	
				cpm/10 mg.	%*	cpm/10 mg.	%*
A	1	leucine 2.2 valine 3.0	5.84	4,110	100	6,380	100
	2	leucine 1.1 valine 1.5	8.58	2,220	68	4,200	71
	3		6.36	3,320		4,780	
	4	leucine 0.55 valine 0.75	5.94	3,690	101	5,160	83
	5		4.64	4,620		5,430	
B	1	leucine 2.2 valine 3.0	5.50	4,600	100	8,150	100
	2		4.99	5,480		9,450	
	3	leucine 0.22 valine 0.30	6.33	1,880	48	2,910	45
	4		5.04	2,920		5,010	
	5	leucine 0.11 valine 0.15	4.84	1,700	31	2,860	30
	6		6.56	1,340		2,380	

* Calculated as % of counts incorporated using maximum C^{14}-amino acid concentrations in each experiment (av. of duplicates).

† The specific activities were: leucine-C^{14}, 7.80 mC./mM; valine-C^{14}, 6.1 mC./mM.

porated into the ASF increased progressively with time of incubation (*i.e.*, at ½ hour, it was 2,740 cpm/10 mg. of tissue; at 2 hours, 10,400; and at 9 hours, 23,500). A similar increase with time was also observed in the TPR fraction. These results clearly indicate that the progressive increase in counts in the ASF is a result of a metabolic incorporation of the added amino acids. They also suggest that few, if any, of the contained counts are due to the adsorption of C^{14}-amino acids.

When the incubations were performed in the absence of oxygen (Table V), incorporation of radioactivity into the ASF was markedly diminished; the inhibition was 80 to 90%. Incorporation into the TPR was also inhibited in the absence of oxygen. Since the conditions of incubation and subsequent fractionation were identical, except in the substitution of 95% N_2-5% CO_2 for the oxygen mixture, these studies clearly indicate that the incorporation of amino acids is oxygen-dependent, and therefore related to an energy-utilizing process.

The original C^{14}-amino acid concentrations selected proved to be greater than that required for maximal labeling of the protein fractions. The effect of varying the concentrations of amino acids is shown in Table VI. When they were decreased to 0.55 m*M*/l. leucine-C^{14} + 0.75 m*M*/l. valine-C^{14} (*i.e.*, one-quarter of the original C^{14}-amino acid concentrations), the level of incorporation into the pro-

TABLE VII

Incorporation of amino acids into protein fractions of goosefish islet tissue: Comparison of leucine-C^{14} + valine-C^{14} of varying specific activities with leucine-H^3

Amino acid concentration (*m*M/l.) Sp. act. (mC/*m*M)	Leucine-C^{14}+Valine-C^{14} 2.1 8.8 1.6 10.5				Leucine-C^{14}+Valine-C^{14} 2.2 7.8 3.0 6.1				Leucine-H^3 .43 3,570			
Fraction	Alcohol-soluble fraction (ASF)		Trichlor-precipitable protein residue (TPR)		ASF		TPR		ASF		TPR	
Time of incubation (hours)	0	2	0	2	0	2	0	2	0	2	0	2
Number of samples	10	21	10	20	11	33	11	32	1	5	1	5
cpm/10 mg. tissue ± S.E.*	211 ±63	5,320 ±681	106 ±25	6,540 ±1,040	305 ±48	5,720 ±420	67 ±18	8,560 ±466	2,200	48,100 ±4,000	204	41,500 ±2,150
Ratio ASF/TPR (2-hour values)	0.81				0.67				1.2			

* Standard errors were calculated using the equation: S.E. $= \sqrt{\frac{\sigma^2}{N}}$ where σ = standard deviation.

tein fractions was essentially unchanged. When they were decreased to 0.22 m*M*/l. leucine-C^{14} + 0.30 m*M*/l. valine-C^{14} (*i.e.*, one-tenth of the original C^{14}-amino acid concentrations), the level of incorporation was decreased by only 50%.

Table VII summarizes the results obtained using mixtures of leucine-C^{14} + valine-C^{14} of varying specific activities with those obtained using leucine-H^3. The counts incorporated per 10 mg. of islet tissue were dependent upon both the specific activities and the concentrations of the amino acids used. The incorporation into the ASF, using tritiated leucine, was 7- to 10-fold greater than that obtained with C^{14}-labeled amino acids. Considering that the counting efficiency of tritium was about 4%, whereas that of C^{14} was 31%, the theoretical number of counts incorporated into the ASF using leucine-H^3 was 60 to 80 times greater than that obtained using C^{14}.

TABLE VIII

Effect of added glucose on C^{14}-amino acid incorporation into goosefish islet tissue protein fractions

Sample no.	Glucose conc. mg./100 ml.	Experiment A					Experiment B				
		mg. tissue	Alcohol-soluble fraction (ASF)		Trichlor-precipitable protein residue (TPR)		mg. tissue	ASF		TPR	
			cpm†	%*	cpm†	%*		cpm†	%*	cpm†	%*
1	0	5.13	5,580	100	14,200	100	4.26	4,600	100	9,050	100
2	12.5	3.39	6,910	124	13,800	97.2	3.79	2,860	84.0	10,600	117
3	25	4.23	5,330	95.6	11,700	82 4	4.76	2,300	50.0	5,720	63.2
4	50	5.25	4,480	80.3	6,790	47.8	4.14	1,780	38.7	4,300	47.6
5	100	4.82	3,260	58.5	3,120	22.0	4.64	1,500	32.6	3,840	42.4
6	200	3.88	2,730	49.0	5,210	36.6	4.54	1,360	29.6	3,530	39.0

The concentration of leucine-C^{14} was 2.2 mM/l., S. A. = 7.80 mc./mM.
The concentration of valine-C^{14} was 3.0 mM/l., S. A. = 6.05 mC./mM.
* Calculated as % of counts found in sample No. 1 without added glucose.
† Expressed as cpm/10 mg. of tissue.

When the counts incorporated into the ASF and TPR fractions were compared, it was observed that the ratio of counts in the ASF/TPR was greatest when tritiated leucine was used, and lowest when the valine-C^{14} concentration exceeded that of leucine-C^{14}. These results suggest that the rate of incorporation of specific amino acids into protein differs for the ASF and TPR fractions.

The effect of adding glucose to the incubation medium is shown in Table VIII. The incorporation of C^{14}-amino acids into both the ASF and TPR fractions decreased progressively with increasing glucose concentration. At a concentration of 100 mg./100 ml., the incorporation was decreased by 40 to 70% of that obtained in samples incubated without glucose.

TABLE IX

Recovery of I^{131}-labeled insulin, added to acid alcohol, followed by precipitation with acetone

Sample no.	Unlabeled insulin μg./100 μl.	Insulin fraction (acetone precipitate)		Acetone supernatant	
		cpm†	% of recovered counts	cpm†	% of recovered counts
1	100	2,420	68.0	1,150	32.3
2	50	1,620	43.9	2,080	56.4
3	20	512	13.8	3,200	86.3
4	10	315	7.8	3,700	92.4
5	5	201	5.1	3,740	95.0
6	2	149	3.9	3,680	96.3
7	1	154	4.0	3,720	96.1
8	0.5	120	3.1	3,760	96.9
9	0	266	7.5	3,290	92.6

† Expressed as cpm/10 mg. of tissue.

Purification of the ASF was accomplished by employing a method based on the solubility of insulin in acid alcohol, and its precipitation by acetone. The ASF was dried and extracted with acetone and ether in order to remove lipids. The residue was dissolved in acid alcohol, centrifuged, and the supernatant transferred to a new tube. It was then precipitated by 4 volumes of acetone, and redissolved in acid alcohol. The efficacy of this procedure was tested by adding tracer amounts of insulin-I^{131} to various concentrations of non-radioactive insulin. It was found that in the presence of 100 μg. of unlabeled insulin per 100 μl. of acid alcohol, approximately 68% of the I^{131} radioactivity was recovered by this purification procedure (Table IX). When smaller amounts of unlabeled insulin were used, a corresponding decrease in recovery was obtained.

Upon purification of the ASF, illustrated in Table X, 65 to 90% of the counts recovered were found in the purified alcohol-soluble fraction; of the total counts recovered, the lipid solvant fraction (LSF) accounted for less than 10%, whereas the acetone supernatant accounted for approximately 10%. The total number of counts recovered in these three fractions varied, in different samples; they averaged 65.2% of the counts added: incomplete recovery would be expected when small amounts of insulin were present.

The protein contents of the purified ASF and the TPR fraction were determined; these values are expressed as μg. of protein per 10 mg. of tissue. The specific activities were expressed as cpm per mg. of protein. The results are given in Table XI.

Approximately 30 μg. of protein were recovered in the purified ASF isolated from 10 mg. of islet tissue. The specific activity of the purified ASF averaged 128,000 cpm per mg. of protein, whereas the specific activity of the TPR averaged 43,000 cpm per mg. of protein. Thus, the specific activity of the purified alcohol-

TABLE X

Purification of the alcohol-soluble fraction obtained from goosefish islet tissue incubated in C^{14}-leucine + C^{14}-valine†

Sample no.	Initial cpm/ 10 mg.	Total counts recovered cpm/ 10 mg.	% recovery	Purified alcohol-soluble fraction		Lipid solvant fraction (LSF)		Acetone supernatant	
				cpm/ 10 mg.	%*	cpm/ 10 mg.	%*	cpm/ 10 mg.	%*
1	7,630	2,920	38.1	2,480	85.8	230	7.9	171	6.43
2	10,500	5,260	50.2	3,440	65.5	1,350	25.6	469	8.92
3	5,890	6,160	105	4,770	77.5	646	10.5	760	12.3
4	4,590	4,740	103	3,900	82.3	448	9.43	400	8.45
5	4,880	3,400	69.8	2,600	76.6	462	13.6	336	9.86
6	4,440	3,520	79.3	2,840	80.6	269	9.48	418	11.9
7	9,740	4,000	41.2	3,580	89.8	269	6.75	146	3.66
8	8,870	4,740	53.5	4,140	87.0	340	7.16	280	5.89
9	13,300	6,750	50.8	5,360	79.5	454	6.74	920	13.7
10	8,660	5,280	61.0	4,320	81.9	520	9.85	435	8.25

* % of total counts recovered.

† The concentration of leucine-C^{14} was 2.2 mM/l., S. A. = 7.80 mC./mM. The concentration of valine-C^{14} was 3.0 mM/l., S. A. = 6.05 mC./mM.

TABLE XI

Specific activities of the purified alcohol-soluble fraction† and trichlor-precipitable protein residue

Sample	mg. tissue	Purified alcohol-soluble fraction			Trichlor-precipitable protein residue (TPR)		
		cpm*	μg. protein / 10 mg. islet tissue	Specific activity cpm/mg. protein	cpm*	μg. protein / 10 mg. islet tissue	Specific activity cpm/mg. protein
1	3.62	7,630	26.8	93,000	9,670	—	—
2	4.40	10,500	20.5	167,000	13,000	144	39,700
3	4.06	5,890	35.4	134,000	8,670	80.0	44,000
4	5.70	4,590	29.0	135,000	8,950	152	33,600
5	4.96	4,880	33.2	78,100	4,800	119	20,000
6	4.46	4,440	34.6	82,400	5,430	113	21,400
7	5.16	9,740	29.4	121,000	10,300	—	—
8	5.38	8,870	30.9	134,000	12,700	122	56,500
9	2.92	13,300	28.0	191,000	19,900	72.3	80,400
10	3.88	8,660	30.0	145,000	10,700	79.3	52,200
Averages ± S.E.**	4.45	7,850	29.9	128,000 ±10,800	10,400	110	43,500 ±6,590

† The purified alcohol-soluble fractions used were the same as shown in Table X.

* The incorporation of amino acid into the protein fraction during a two-hour incubation period is expressed as cpm/10 mg. of islet tissue.

** The standard errors were calculated as in Table VII.

soluble fraction is approximately three times greater than that of the trichlor-precipitable protein residue.

DISCUSSION

The results obtained clearly indicate that C^{14}- and H^3-labeled amino acids, incubated *in vitro* with islet tissue, are incorporated into the protein fractions. Since added I^{131}-labeled insulin is recovered in the purified alcohol-soluble fraction, it is presumed that insulin synthesized *in vitro* by islet tissue will similarly be found in this fraction. Although the total counts incorporated into the alcohol-soluble fraction are approximately equal to that incorporated into the trichlor-precipitable protein fraction, the specific activity of the alcohol-soluble fraction is three times greater than that of the trichlor-precipitable protein residue. The incorporation of C^{14}-amino acids into the alcohol-soluble fraction is increased with increasing periods of incubation. It is markedly inhibited when the incubations are carried out in the absence of oxygen, thus suggesting that the incorporation is dependent upon an active metabolic process which requires energy. The incorporation is also dependent upon the amino acid concentration; however, maximal values are reached at leucine and valine concentrations of about 0.25 m*M*/l.

The addition of glucose decreases the counts incorporated into both the alcohol-soluble fraction and trichlor-precipitable protein residue. A similar effect of glucose on the incorporation of alanine-C^{14} into TCA-insoluble protein was observed by Sinex *et al.* (1952). Since hyperglycemia stimulates the release of insulin from the β-cell (Lazarow, 1960), it would have been expected that glucose might

increase the rate of insulin secretion. This apparent inhibitory action of glucose might be explained by the appearance of intermediates (derived from glucose) which can be used for the synthesis of insulin; this hypothesis requires further study.

The specific activities of the ASF proteins obtained in the C^{14} studies (averaging 128,000 cpm/mg. of protein) and in the tritium studies (400,000 cpm/mg. of protein) are many times greater than those previously reported using mammalian pancreas incubated *in vitro*. For example, Pettinga and Rice (1952) isolated insulin containing 1600 cpm/mg. of protein; in the studies of Light and Simpson (1956), the specific activities were less than 1000 cpm/mg. of protein.

In order to further characterize the radioactive protein found in the ASF, additional studies have been done using a specific immuno-precipitation method. Under the appropriate conditions, serum prepared from guinea pigs previously immunized to bovine insulin will form a specific insulin-antibody complex. Using the method of Skom and Talmage (1958), this soluble complex may be precipitated by reacting it with anti-guinea pig serum obtained from rabbits immunized to normal guinea pig serum. Studies carried out by Carl R. Morgan (personal communication) using insulin-I^{131} as a marker, have indicated that approximately 85% of added insulin-I^{131} (1 μg.) combines with the specific insulin-antibody and is precipitated by the anti-guinea pig serum (rabbit). Control studies, identical to the above, except for the substitution of normal guinea pig serum for the anti-insulin guinea pig serum, yielded insignificant counts in the precipitated complex.

Similar immuno-precipitation experiments have been carried out using the tritium-labeled goosefish alcohol-soluble fraction (specific activity = 400,000 cpm/mg. of protein); these strongly support the thesis that a significant fraction of the tritium label found in the alcohol-soluble fraction represents insulin which has been synthesized *in vitro* from added leucine-H^3.

The counts incorporated into the alcohol-soluble fraction of islet tissue can be expressed as mμ moles of amino acid per gm. of islet tissue per hour. In the leucine-H^3 experiment (where the amino acid concentration was 0.43 mM/l., specific activity = 3,570 mC./M, and the counting efficiency assumed to be 4%), an incorporation of 48,100 cpm/10 mg. of tissue during a two-hour period is equivalent to 7.5 mμ moles of leucine per gm. of islet tissue per hour. Since 6 of the 51 amino acid residues in insulin are leucine, the maximal rate of insulin synthesis would not be greater than 1.25 mμ moles of insulin per gm. per hour. Converting this to units per day, the maximal rate of insulin synthesis would not be greater than 4.5 units per gm. of islet tissue per day. The human pancreas contains approximately 1 gm. of islet tissue (Lazarow, 1960), and it has been reported that the totally depancreatized man can be maintained with doses of insulin as low as 20 units per day (Rockey, 1943; Goldner and Clark, 1944). It should be noted that the *in vitro* incubation studies using goosefish islet tissue were carried out at 25° C. (whereas insulin synthesis in man takes place at 37° C.) and that the rate of chemical reactions doubles with each 10° increase in temperature. Thus, the calculated maximal rate of insulin synthesis *in vitro*, based on the observed rate of amino acid incorporation into the alcohol-soluble fraction and corrected for temperature, would approximate 10 units per gm. per day; this approaches the order of magnitude of the physiologic rate of insulin synthesis by human islet tissue.

Summary

1. Goosefish islet tissue, incubated *in vitro* with C^{14}- or H^3-labeled amino acids, showed significant labeling of the protein fractions.

2. The purified alcohol-soluble fraction is presumed to contain the insulin which is synthesized *in vitro*.

3. The rate of amino acid incorporation into the alcohol-soluble fraction increases progressively with increasing time of incubation; it is decreased in the absence of oxygen.

4. The addition of glucose decreases the number of counts incorporated into the alcohol-soluble fraction.

5. In the C^{14}-amino acid incorporation experiments, the specific activity of the purified alcohol-soluble fraction (128,000 cpm/mg. protein) is three times greater than that of the trichlor-precipitable protein residue.

6. These studies support the thesis that amino acids, added to the islet tissue *in vitro,* are incorporated into insulin.

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